(R)-Albuterol Elicits Antiinflammatory Effects in Human Airway Epithelial Cells via iNOS

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METHODS

Treatments

Treatments including 10 or 50 ng/ml each of tumor necrosis factor-α (TNF-α; R&D Systems, Minneapolis, MN), interleukin-1β (IL-1β), and/or interferon-γ (IFN-γ) were prepared in warmed (37°C) serum-free BEBM medium before application to cell culture surfaces. (R)-, (S)-, or racemic albuterol (provided by Sepracor Inc., Marlborough, MA) were added in selected studies. Racemic mixtures were prepared by combining equimolar amount of (R)- and (S)- albuterol prior to treatment, i.e., 10^{-6}M racemic albuterol was composed of 10^{-6}M (R)-albuterol and 10^{-6}M (S)-albuterol. For inhibitor studies, pharmacological inhibitors were added 0-30 min prior to cytokine/albuterol administration. Inhibitors for cAMP-dependent kinase (H-89; $IC_{50} < 50$nM) (E1), protein kinase C (calphostin C; $IC_{50} = 50$nM) (E2), protein kinase Cδ (rottlerin; $IC_{50} = 3$-6μM) (E3), protein kinase Cε (translocation inhibitor peptide; Calbiochem, La Jolla, CA), cGMP-dependent kinase (8-[4-cholorophenylthio]-guanosine 3',5'-cyclic monophosphorothioate, Rp Isomer triethylammonium salt, also known as (Rp)-8-pCPT-cGMPS; $IC_{50} = 0.5$μM) (E4), and β_2-adrenergic receptor (erythro-D,L-1[7-lethylindan-4-yloxy]-3-isopropylamino-+++butan-2-ol, also known as ICI 118,551; $IC_{50} = 15$nM) (E5) were used. To test for PKC mediated activation of iNOS expression, the general PKC activator phorbol 12-myristate 13-acetate (PMA), and the PKCδ/ε isozyme activator, bryostatin 1, were used at concentrations previously published (E6, E7). An inert PMA analogue, 4-α-PMA, was used as a negative control. All reagents, unless otherwise noted, were purchased from Sigma-Aldrich (St. Louis, MO).

For some time-course studies, reagents of interest were co-incubated with IL-1β and IFN-γ for 6 or 8 hrs, as noted. For some studies requiring shorter exposures (e.g., 30 min, 1 hr, etc.)

agents were added to the cytokine-exposed culture medium so that the experiments were stopped simultaneously with the appropriate IL-1β and IFN-γ exposure time.

Some cells were lysed and lysates collected for total protein determination or assayed for PKC activity. Briefly, medium was vacuum-aspirated from cultures and then washed once with phosphate-buffered solution (PBS). Cells were then scraped into lysis buffer (~50 µl volume/well) containing 50mM Tris-HCl [pH7.5], 1mM EDTA, 100mM NaCl, 5mM $MgCl_2$, 10mM dithithreitol (DTT), 1mM phenylmethylsulfonyl fluoride (PMSF), with 10% (v/v) protease inhibitor cocktails (P-8340, P-2850; Sigma, St. Louis, MO).

TaqMan Real-time Reverse Transcriptase Polymerase Chain Reaction

Total RNA was isolated from cell lysates using an RNeasy Mini Kit according to the manufacturer's instructions (Qiagen, Valencia, CA). Each sample was treated with RNase-free DNase for 15 minutes to reduce DNA contamination. Total RNA was then quantified and checked for purity by measuring A_{260}/A_{280} ratio on a UV spectrophotometer, and only those with ratios above 1.95 were used for reverse transcription. 1 µg of RNA from each sample was reverse transcribed using an iScript cDNA synthesis kit according to recommended thermal conditions (Bio-Rad, Hercules, CA).

Real-time PCR was performed using a hydrolysis (TaqMan) probe. 0.5 µl of each reverse-transcribed sample was added to a 25 µl reaction volume containing 200 nM forward and reverse primers, 50 nM dual-labeled nonextendable oligonucleotide hydrolysis (TaqMan) probe, 1X Absolute QPCR Mix containing DNA polymerase (ABGene, Epsom, Surrey, UK), and nuclease-free water in a thin-walled 96-well reaction plate (BioRad, Hercules, CA). Amplifications for each sample were performed in triplicate on an iCycler iQ Real-Time PCR

Detection System (BioRad) with the following cycling conditions: 15 min 95°C *Taq* polymerase activation and 45 cycles of amplification [15 sec 95°C denaturing cycle, 1 min 58°C annealing/extending]. Both target and β-actin amplifications were performed on the same plate to negate plate-to-plate variability. Each sample was run in triplicate and amplifications without template were run as negative controls.

Primer and probe sequences were designed using Beacon Designer software (Premier Biosoft, Palo Alto, CA) based on published mRNA sequences for human iNOS, GM-CSF, and β-actin obtained from the NCBI sequence online database (http://www.ncbi.nlm.nih.gov). Forward and reverse primer sequences for each target were located on separate exons to reduce the possibility of amplifying contaminating DNA in samples. Table E1 lists the target sequence GI accession number, primer and probe sequences, and amplicon length and location. Single amplification products void of primer-dimer formation for each target were verified using SYBR Green (BioRad) melt-curve analysis. Hydrolysis (TaqMan) probes were dual-labeled with 6-carboxy-fluorescein (FAM) as a fluorescent reporter dye, and Black Hole Quencher-1 (BHQ-1; Intergrated DNA Technologies, Coralville, IA) as a quencher.

Relative quantification of targets, normalized to β-actin message, was achieved according to a method described by Peirson, *et al* (E8), which estimates initial fluorescence (R_o) value of each individual amplification based on estimated amplification efficiency and measured fluorescence at the amplification threshold cycle (R_{Ct}). The resulting target R_o value for the target amplification of each treated sample is normalized to the R_o value of β-actin for the same sample. This normalized value is then expressed as a ratio of the normalized R_o value of a control sample.

NHBE Transfection and RNA Silencing

NHBE cells were grown in 12-well plates in growth medium void of antibiotics until ~60-75% confluent. Medium was then replaced with 450 µl (volume) of medium without antibiotics, bovine pituitary extract and human recombinant epidermal growth factor, and the cells incubated at 37°C/5% CO_2 until transfected. To prepare the transfection mixture for an individual culture well, 2 µl of Fugene 6 transfection reagent (Roche Diagnostics Corp., Indianapolis, IN) was added directly to 48 µl of serum- and antibiotic-free media without touching reagent to the plastic walls of the tube. After brief vortexing at room temperature for 5 min, 0.1 nmole annealed small interfering RNA (siRNA) for human iNOS (catalog #8678; Ambion, Austin, Texas) was added to the transfection preparation, vortexed briefly, and incubated at room temperature for 15 mins. After incubation, 50 µl transfection complex was then added to each culture well to a final concentration of 0.45% Fugene and 225 nM iNOS siRNA and returned to 37°C/5% CO_2 for 24 hrs. These concentrations were experimentally optimized to elicit the greatest iNOS message silencing in NHBE cell cultures (~60%) without adverse cellular effects. As a control, some cultures were transfected with scrambled siRNA (catalog #4611, Ambion) or Fugene 6 reagent alone. At 0.45% final concentration, Fugene 6 transfection reagent did not alter iNOS, GMCSF, or β-actin message levels from non-treated cultures. Scrambled siRNA did elicit some knockdown of iNOS message (~30%, see figure E1). However, iNOS siRNA consistently elicited a greater knockdown. After 24 hr. transfection incubation, cultures were treated after serum- and antibiotic-free media change for no longer than 24 hrs post-transfection.

SUPPLEMENT REFERENCES

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Table E1. Primer and Probe Sequence Information

Name (Accesion #)	Human Inducible Nitric Oxide Synthase (GI#24041028)	Human Granulocyte Macrophae Colony Stimulating Factor (GI# 27437029)	Human B-actin (GI# 5426604)
Forward Primer	5'/GCCGCATGATTTCCTTGGTG/3'	5'/CTCAGAAATGTTTGACCTCCAGGA/3'	5'/TTGAATGATGAGCCTTCGTGCC/3'
Reverse Primer	5'/CCATCTCCAGCATCTCCTCCTG/3'	5'/CTTGAGCTTGGTGAGGCTGC/3'	5'/TGGTCTCAAGTCAGTGTACAGGTA/3'
Probe	5'/FAM/TGCCGCCGCCCAGATGAGGACC/BHQ-1/3'	5'/FAM/CCGACCTGCCTACAGACCCGCCTG/BHQ-1/3'	5'/FAM/CCTGGCTGCCTCCACCCACTCCC/BHQ-1/3'
Amplicon Length (bp) and Location	75 [3287-3361]	94 [212-305]	130 [1642-1772]

Figure E1

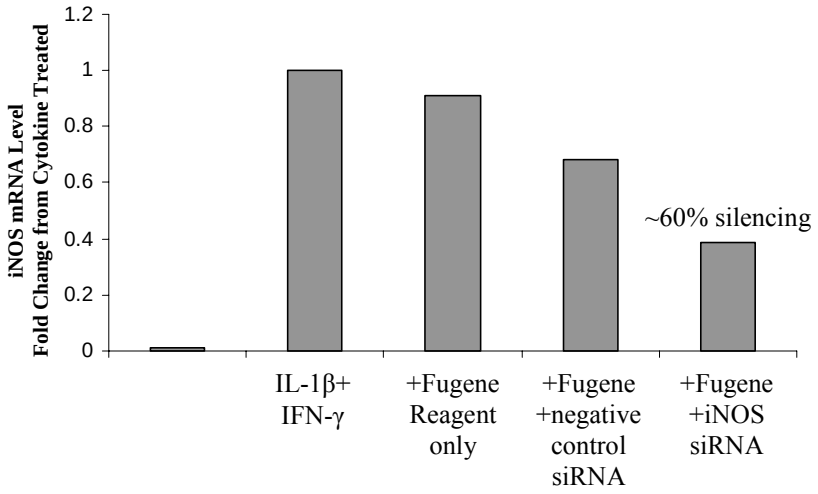

Figure E1. siRNA effectively decreases iNOS mRNA expression. NHBE cell cultures were transfected with iNOS silencing RNA using Fugene 6 reagent. Cells were transfected with a 0.45% final concentration of Fugene reagent and 225 nM iNOS small interfering RNA or scrambled siRNA control (Ambion) for 24 hrs. in growth medium void of antibiotics. After this transfection period, cells were stimulated with 10 ng/ml each of IL-1β+IFN-γ for 18 hrs. to induce iNOS transcription. iNOS message, normalized to β-actin message, was assayed by TaqMan real-time RT-PCR after total RNA isolation. Fugene transfection reagent alone did not modulate iNOS induction. Addition of the iNOS siRNA reduced cytokine-induced iNOS transcription by ~60%. Some attenuation of message was noted with scrambled siRNA.